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Journal of Advanced Veterinary Research
Volume 4, Issue 3 (2014) 102-107 **Original Research**

In vitro Adoption and Propagation of High Pathogenic Avian Influenza (HPAI) Virus Subtype H5N1 in non-avian Host System

Maheswarappa Gowrakkal[1*], Byregowda S.M.[1], Pradhan H.K.[2], Tosh C.[2], Pattnaik B.[2], Renukaprasad C.[1]

[1]*Institute of Animal Health and Veterinary Biologicals, KVAFSU, Hebbal, Bangaluru, India*
[2]*High Security Animal Disease Laboratory (HSADL), Indian Veterinary Research Institute (IVRI), Bhopal (MP), India*

Accepted 26 June 2014

Abstract

The paradigmatic, fatal and devastating ailment called avian influenza or bird flu is a highly contagious viral disease caused by type A influenza virus, It primarily affects the respiratory, digestive and/or nervous system of chickens, turkeys, guinea fowls and other avian species and less commonly pigs and other species of mammals including human. In India, The first pandemic outbreak of Avian Influenza was reported during 2006. In this study, we selected an isolate of high pathogenic avian influenza (A/Ck/Jalgaon/India/12419/2006) H5N1 virus and propagated in chicken embryo fibroblast. Later this virus was adopted and propagated in Madin-Darby canine kidney cells (MDCK) and Vero cells. Infected non-avian cells with an avian virus shown cytopathic effects like rounding, cytoplasmic elongation, syncytia formation and later stages fluffing from the attached surface. The harvested virus suspension shown increased haemagglutination titre (HA) than viral suspension from chicken embryo fibroblast culture and the presence of virus was confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR). The obtained result reveals that virus had capacity to adopt for the invitro culture and propagate in non avian host cells with higher titre. This infers the chance of virus to cross the host barrier and probable chance of infection in human being.

Keywords: AIV; CEF cells; MDCK cells; Vero cells; Polymerase chain reaction; Host barrier.

Introduction

Influenza viruses belong to the family Orthomyxoviridae, classified as types A, B and C genus on the basis of nucleoprotein (NP) and matrix protein (M1) and have segmented, single-stranded, and negative-sense RNA genome in an enveloped virion (Webster, *et al*., 2002). Avian influenza is caused by type A influenza virus, which is further classified into subtypes on the basis of two surface glycoproteins; hemagglutinin and neuraminidase. Avian Influenza viruses had wide host range and Migratory water birds' acts as main reservoir host and play key role in spreading of virus to other hosts (Chen, *et al*., 2006).

A number of cell/ tissue culture systems (Taubenberger and Layne, 2001) have been set up to isolate influenza A viruses. Avian influenza viruses replicate in a limited number of cell cultures. Chicken embryo fibroblasts (CEF) are the most commonly used primary cell cultures, where as the frequently used continuous cell line is the Madin-Darby canine kidney (MDCK) cells and is the currently accepted 'Gold-standard' for laboratory diagnosis of influenza virus (Gavin and Thomson, 2003). After culture isolation, most influenza isolates are definitively identified using immune assay or immunofluorescence test (Murphy and Webster, 1996). However traditional virus isolation and identification takes times and virus culture results are generally available in four to five days followed by primary rhesus monkey (PMK) cells (Gavin and Thomson, 2003). Influenza virus was adopted and propagated in Vero cells (Kamal, *et al*., 2006).

*Corresponding author: Maheswarappa Gowrakkal

E-mail address: maheshkathige@gmail.com

ISSN: 2090-6277/2090-6269, www.advetresearch.com

Materials and methods

The H5N1 virus isolate (A/Chicken/12419/Jalgaon/India/2006) was propagated in 9-11 day old fertile chicken eggs. The inoculated embryos were incubated at 35°C for 2-3 days. The embryos that died within 24 h after inoculation were discarded. After 72 hours incubation period the embryos were removed from the incubator and chilled at 4°C (SPF embryonated eggs were obtained from VHL, Pune, INDIA).

Harvesting of Virus from Inoculated Chicken Eggs

Eggs were chilled at 4°C overnight before harvesting. Using a 10 ml pipette, aspirated the allantoic fluid in a labeled plastic tube. The harvested allantoic fluid was placed in a separate tube, Harvested fluids was centrifuged at 3000 rpm for 5 min to remove blood and cells. The clear supernatant was aseptically transferred into sterile and labeled vials. These vials were then stored at -70°C until further use.

Cell culture study to check the cytopathic effect of virus

After the embryo passage, the virus was further adapted to MDCK cell line. The cells were maintained in GMEM with regular sub culturing (Cell lines were obtained from National center for cell science, Pune, India).

Infection of MDCK Cell Line

The MDCK cells were sub cultured into 24 well cell culture plates and incubated overnight. When the monolayer was 60- 80%, the monolayer was washed twice with the GMEM medium and 150 µl of the serially diluted virus from 10^{-1} to 10^{-5} was dispensed into the wheel C 1-6 and D 1-6. The cells were incubated for 1hour in a CO_2 incubator at 37ºC for virus adsorption (INNOVA 4080, New Brunswick Scientific, India). After 1 hour the cells were washed thrice with GMEM medium and 1 ml of GMEM medium was added to the wells C 1-6 (Virus control) and 1 ml of GMEM with trypsin (0.5 µg/ml) was added to the wells D 1-6. About 1 ml of GMEM was added to the wells A 1-6 (Cell control) and 1 ml of GMEM with Trypsin (0.5 µg/ml) was added to the wells B 1-6 (Trypsin control). The plate was incubated at 37ºC for 3 days and observed daily twice for the development of cytopathic effects. Later virus infected cell suspension was harvested and stored at – 80°C for further use. Same procedure was followed to check the cytopathic effect in chicken embryo fibroblast (CEF) cells and Vero cells. After complete cytopathic effect, virus infected cell suspension was harvested and stored at – 80°C for further use.

Haemagglutination (HA) Test

Preparation of 1% (v/v) Chicken RBCs

Five milli liter of chicken blood was collected in 5 ml of Alsever's solution in a 10 ml syringe. To remove the Alsever's, RBC's were washed three in 1X PBS (pH 7.2) and the pellet was resuspended in 1X PBS to a final concentration of 1% .

Haemagglutination (HA) Test

Into each well of a plastic V bottomed micro titer plate 25 µl of 1X PBS was dispensed. In the first well of the row 25 µl of allantoic fluid from embryonated chicken eggs inoculated with virus was dispensed and serially diluted to two fold dilutions made from 1:2 to 1:4096. Further 25 µl PBS was dispensed to each well. Then 25 µl ml of 1% (v/v) chicken RBCs was dispensed to each well. One row of well was kept as RBC control, which was

Table 1. Sequence of oligonucleotide primers used in the study

Primer Description	Sequence (5'-3')	Source
Lee - FP	ACACATGCYCARGACATACT	Lee *et al.*, 2001
Lee - RP	CTYTGRTTYAGTGGTTGATGT	Lee *et al.*, 2001
WHO - FP	GCCATTCCACAACATACACCC	WHO 2005
WHO - RP	CTCCCCTGCTCATTGCTATG	WHO 2005

prepared by dispensing 50 µl of PBS and 25 µl ml of 1% chicken RBCs. After tapping the plate gently for uniform mixing the plate was incubated for 30 min at the room temperature (20-25^{o}C). The HA titre was determined by tilting the plate and observing the presence or absence of tear shaped streaming of the RBCs against the RBC control. The reciprocal of the highest dilution giving complete HA (no streaming) is taken as HA titre. One HAU is the amount of Heamagglutinin contained in the end point dilution of the HA titration.
Simillarly, HA titre was determined to viral suspension obtained from harvested chicken embryo fibroblast cells, MDCK cells and Vero cells. Then obtained HA titers of same H5N1 virus of three different sources were compared.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from infected allantoic fluid using QIAamp Viral RNA Mini Kit (Qiagen, Germany). One-step Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was done for HA gene amplification using AMV-RT enzyme kit (Promega, USA).

The reaction volume of 25 µl was used for the other RT-PCR reaction with different sets of primers viz. WHO-F, WHO-R and Lee-F, Lee-R. The reactions were carried out in a thermal cycler (Perkin Elmer, USA) as per the following temperature cycles:
Step I: Reverse Transcription :48^{o}C for 30 min (1 cycle)
Step II: Initial denaturation: 94^{o}C for 2 min (1 cycle)
Step III: Denaturation : 94^{o}C for 30 s
Annealing of primer: 50^{o}C for 30 s (35 cycles)
Extension: 68^{o}C for 40 s
Step IV: Final extension 68^{o}C for 10 min (1 cycle)

Similar reaction was used for RT-PCR amplification with same primers but RNA was extracted from infected CEF cells, MDCK cells and Vero cells. The PCR amplified products were checked in 1% agarose gel with 1000 bps ladder using gel documentation system (Syngene).

Results

The HA test performed using 1% of chicken RBCs reveals that all four samples such as virus infected allantoic fluid, cells suspension of CEF, MDCK and Vero infected with H5N1 virus shown HA titre. Negative control where in virus suspension was not used shows no lattice formation with chicken RBCs and positive control with neat virus suspension without dilution with PBS shown clear lattice formation during HA titration. The Haemagglutination titre results were 2^{6}, 2^{8}, 2^{9} and 2^{8} for viral suspension of infected allantoic fluid, chicken embryo fibroblast cells, MDCK cells and Vero cells respectively.

Discussion

The Haemagglutination titre results were 2^{6}, 2^{8}, 2^{9} and 2^{8} for viral suspension of infected allantoic fluid, chicken embryo fibroblast cells, MDCK cells and Vero cells respectively. This titre values indicated that virus could propagate faster in cell culture than embryonated eggs and clearly reveals that H5N1 virus has adapted rapidly to nonavian host species. HA titre of 2^{9} in MDCK cells could be due

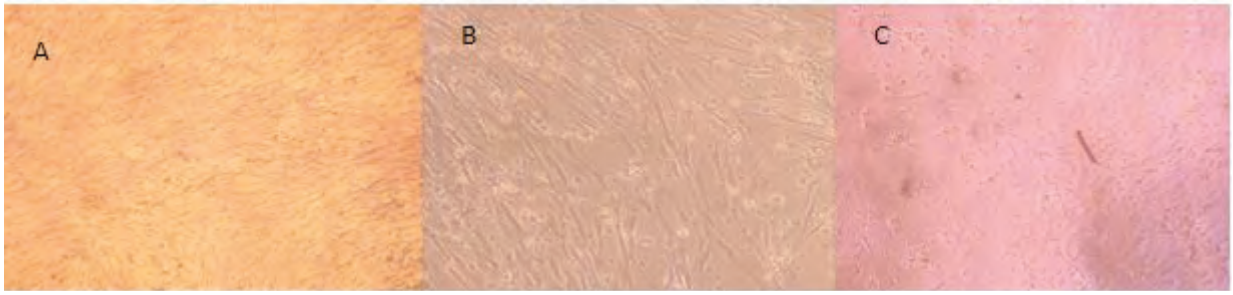

Plate 1. CEF cells infected with H5N1 Virus showing cytopathic effects (100X)
a.Normal CEF cells showing confluent monolayer.
b.CEF cells Infected with AIV showing CPE with rounding of cells after 24 hours PI.
c.CEF cells showing extensive cytopathetic changes (CPE) with detachment of cells from the surface of culture plate after 48 hours of infection.

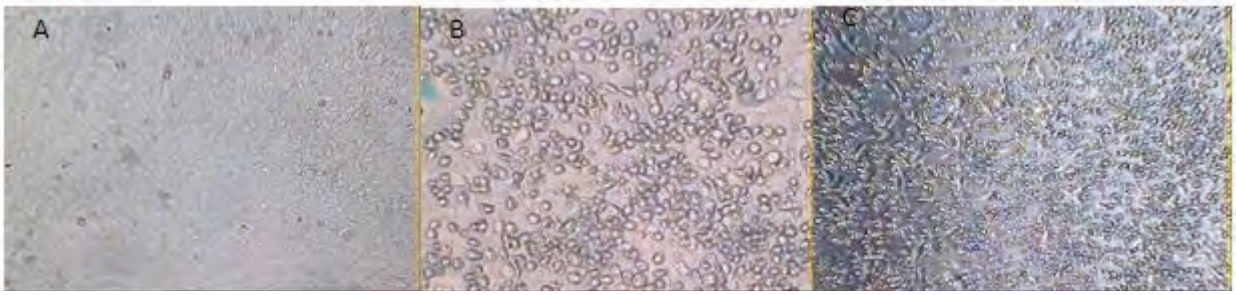

Plate 2. MDCK cells infected with H5N1 Virus showing cytopathic effects (100X)
a.Normal MDCK cells showing confluent monolayer.
b.MDCK cells Infected with AIV showing CPE with rounding of cells after 24 hours PI.
c.MDCK cells showing extensive cytopathetic changes (CPE) with detachment of cells from the surface of culture plate after 48 hours of infection.

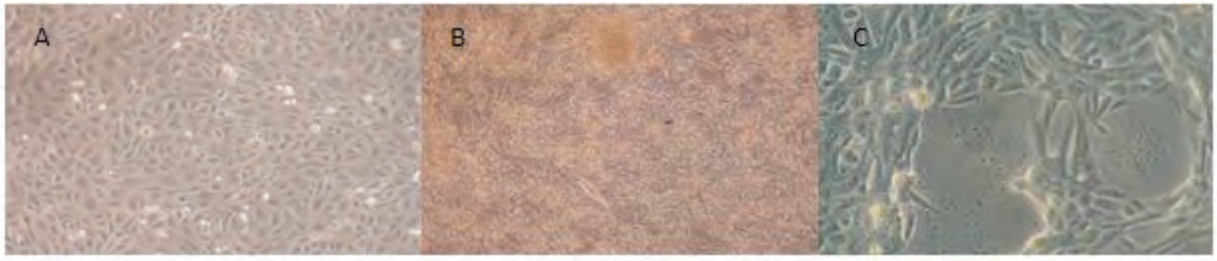

Plate 3. Vero cells infected with H5N1 Virus showing cytopathic effects (100X)
a.Normal Vero cells showing confluent monolayer.
b.Vero cells Infected with AIV showing CPE with rounding of cells after 24 hours PI.
c.Vero cells showing extensive cytopathetic changes (CPE) with detachment of cells from the surface of culture plate after 48 hours of infection.

to virus had capacity to cross host barrier and adapt to non avian host. This higher titre values also caution us that virus could to cross host barrier and infect to human being. This virus is segmented RNA virus which favors genetic reassortment and leads to development new strains, since India had endemic report of low pathogenic avian influenza virus (LPAI) (Nagaraja, *et al*., 2009). There is a chance of mixing of H5N1 and H9N2 virus in natural condition due to congenial environmental condition and close contact of different species of animals with birds including human beings.

Several *In vitro* and in vivo studies were carried o host adaptation of HPAI viruses. Katz *et al*. (2000) studied the Molecular Correlates of influenza A H5N1 virus pathogenesis in mice and molecular changes associated with the transmission of avian influenza A H5N1 and H9N2 viruses to humans were studied by Shaw et al. (2002). Study of cytopathic effect (CPE) using chicken embryo fibroblast cell line indicated that H5N1 virus isolate used in this study was highly pathogenic nature and which start showing cytopathic effect such as rounding of cells from 24 hours of post infection. This is followed by complete fluffing from attached surface at 48 hours of post infection (Plate.1-3). Study of CPE using MDCK cell line indicated that isolate used in study was highly pathogenic in nature with exhibiting complete cytopathic effects 48 hours of Post Infection and well adapted to MDCK cell lines. Cytopathic effects range from rounding of cell, plaque formation to compete detaching of monolayer from surface which clearly indicated that viruses have affinity to infect non-avian species (Hatta *et al*., 2001 and Kamal *et al*., 2006). In this study also found that MDCK cells infected with H5N1 virus showed CPE of rounding, cytoplasmic elongation, syncytia formation and later stages fluffing from the attached surface. Similar CPE were found in Vero cells within 48 hours of post infection. These CPE were indicate that virus has capacity to cross host barrier and adapt to non avian host and all these changes were well indicate the adaptability and multification of H5N1 virus *In vitro* conditions in non avian host system. In similar study, Kim *et al*. (2005) revealed that respiratory tract and digestive tract of avian species have receptors for both avian and human influenza viruses which could favors easy adaptation to other hosts but target different cell types in cultures of human

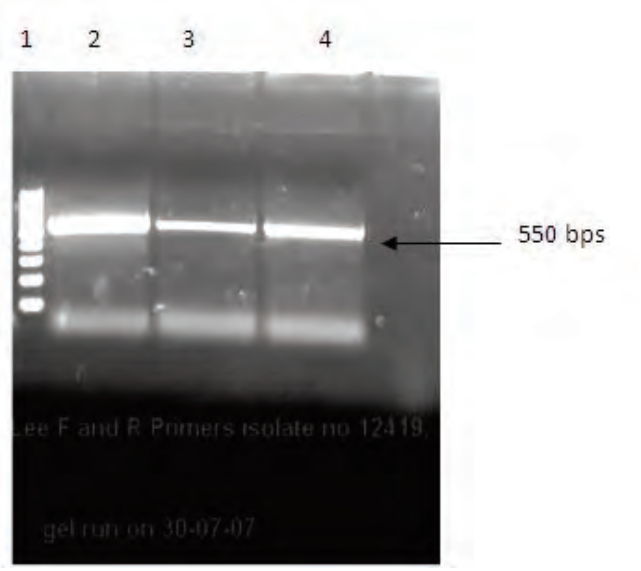

Fig. 1. HA gene amplicons of H5N1 AIV as visualized by Agarose gel electrophoresis. 1:1000 bps ladder. 2,3,4: HA gene amplified products using Lee primers of RNA extracted from CEF cells, MDCK cells and Vero cells suspension respectively.

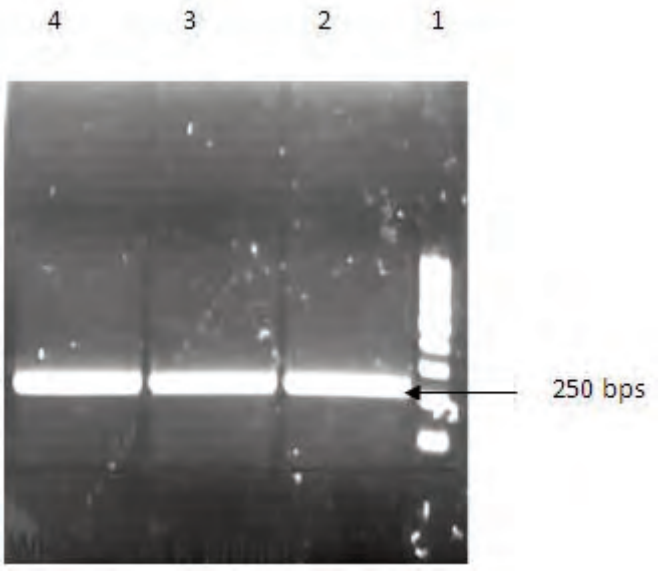

Fig. 2. HA gene amplicons of H5N1 AIV as visualized by Agarose gel electrophoresis. Lane 1: 1000 bps ladder. Lane 2, 3, 4: HA gene amplified products using WHO primers of RNA extracted from CEF cells MDCK cells and Vero cells suspension respectively.

airway epithelium (Matrosovich *et al*., 2004). Another study by Xu *et al*. (1999) on genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus shown similarity of hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong.

Viral Suspensions, which were obtained from infected CEF, MDCK cells and Vero cells, were used for RNA extraction. Then total RNA extracted by Qiagen kit method and the yield was quantified by nanodrop method. The RNA samples were subjected for RT reaction and PCR amplification using the primers specific to each segment of RNA (WHO and Lee primers). Here, amplified the HA segment of AIV using internationally recommended primers. The amplification after the RT reaction was proper and the sizes of the amplified products were of expected size. In all three RNA samples give same size products of 250 bps with WHO primers (Fig.1) and 550 bps with Lee primers which were designed to amplify HA gene of H5 subtype of avian influenza virus (Fig. 2). Avian influenza virus which was known to adapt to other non avian host had more fatal effects (Smith *et al*., 2006) and Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus had devastating nature and quick adaptation to other host (Class *et al*., 1998 and De Castro *et al*., 2006). The three dimensional structure of a complex influenza virus neuraminidase protein provide better host adoptability (Colman *et al*., 1987). Cytopathetic changes in MDCK cells and Vero cells indicated that virus has chance to cross the species barrier and infect non avian host species. The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host (Gabriel *et al*., 2005) and PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 influenza A viruses in mice (Shinya *et al*., 2004).

Conclusion

Human infections and death due to avian influenza virus subtype H5N1 in Hong Kong in 1997 focused global attention on the avian influenza viruses. Subsequently, human diseases associated with H9N2 viruses were also documented which suggested that the other avian influenza viruses can also cross the species barrier to humans. Infections with AIV (H9N2) and outbreaks of highly pathogenic avian influenza due to H5N1 have been reported in India. A high potential for antigenic and genetic variation due to error-prone replication and antigenic shift among the field isolates underscores the need for monitoring the field situation regular intervals. HA titres values of H5N1 isolates indicated that virus could be well propagated in *In vitro*

condition than in vivo condition with higher multification rate. Cytopathetic changes in MDCK cells and Vero cells indicated that virus has chance to cross the species barrier and infect non avian host species. Amplification of HA gene from three different source of virus indicated that highly pathogenic avian influenza virus could be propagated well in both avian and non avian species, which indicate the risk of human infection and zoonotic importance of virus and encourage us to development of better effective therapeutics to curb the future outbreaks.

Acknowledgement

The Author was grateful to Honable Vice Chancellor, Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar. Karnataka and the Director, Indian Veterinary Research Institute, Izatnagar, U.P. for providing necessary facilities this research work.

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